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Public Health Service

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MEMORANDUM

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SUBJECT: Statistical Analysis of the Effects of Perchlorate on Neurobehavior (Motor Activity) in SD Rats

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This memo addresses your request for statistical analysis of the available neurobehavioral data on the effects of ammonium perchlorate administered orally via drinking water to rats. The available data include the study performed by Argus (1998) and the more recent study performed by the United States Navy (Bekkedal et al., 2000).

Background:

In its 1998 risk assessment, the U.S. Environmental Protection Agency (EPA) found a dose-dependent increase in male rat pups on post natal day 14 (PND14) for two different measures of motor activity: (1) time-spent-in-movement ("time") and (2) total number of movements ("movements") as shown in Figure 1 (U.S. EPA, 1998). The EPA had a number of concerns with the Argus (1998) study, noting the lack of appropriate historical control data for the PND14 pups and a high degree of within-group variability in the data exemplified by coefficients of variation (CV) greater than 100%. The latter was posited as a potential explanation for the failure of the statistics performed by the original contactor (Argus, 1998; York, 1998) to find any effect of treatment despite a 95%

increase over controls at the highest dosage group for the time-spent-in-movement (group means of 363 versus 186) and total-number-of-movements variable (65% relative to controls) in that study (Crofton et al., 1998). The expert opinions of several EPA neurotoxicologists was that an increase in a motor activity indicator over 50%, especially in developing animals, was clearly of concern from a biological perspective (Crofton et al., 1998). EPA maintained that the increase in activity seen in the Argus (1998) study should be considered biologically significant until additional data could be marshaled to suggest or prove otherwise (U.S. EPA, 1998). In response to recommendations for an additional study, the United States Navy (USN) performed a study that included evaluation of motor activity in Sprague Dawley rats of both sexes (Bekkedal et al., 2000).

Bayesian Hierarchical Modeling Approach:

In these studies, motor activity was characterized using a battery of items thought to measure different aspects of each animal's movement intensity at each testing age. These items included measurements of the time ambulatory and the time spent performing stereotypic movements. The total distance traveled and the numbers of stereotypic, ambulatory, horizontal, vertical and rearing movements were also noted. After appropriate transformation, each of the outcomes were approximately normally distributed.

There was a very high level of correlation between the different outcome measures, and we therefore focused our analysis on the number of ambulatory movements. We did not analyze the time spent in movements as done by the EPA in 1998 because of this correlation.

We chose to use a Bayesian hierarchical model (Gelfand et al., 1990) to assess the weight of evidence of a dose-response trend in motor activity. A linear mixed-effects regression model (Laird and Ware, 1982) related dose, sex, age, habituation time and a habituation time x dose interaction term to the expected number of ambulatory movements, with an animal-specific intercept included to account for within-animal dependency. To complete a Bayesian specification of our model, we choose vague (or uninformative) but proper prior distributions for each of the unknown parameters. In particular, the prior for the parameters that related dose to motor activity was centered on a value corresponding to the null hypothesis of no effect of perchlorate. Such an approach to specifying the prior has been applied previously in similar health risk assessment applications (Hasselblad and Jarabek, 1996). The model was fit using BUGS, a widely-used software package for Bayesian analyses (Gilks et al., 1994).

To perform a combined analysis of data from the USN Study (Bekkedal et al., 2000) and the Argus (1998) study, we used a modification of the model described above. We first standardized the number of ambulatory movements by substracting the overall mean and dividing by the standard deviation. We then fit a linear mixed-effects regression model that incorporated distinct baseline parameters (i.e., intercept, age-effects, habituation time effects, error variances) for the two studies, but assumed common slope parameters. This model is described in Appendix A.

Analyses were conducted under a variety of different choices of prior variance for the dose parameters and prior means and variances for the other parameters in the model. We estimated the dose level associated with a 10% increase in the number of ambulatory movements by inverse estimation (refer to Appendix A). The choice of 10% as the benchmark level is consistent with standard practice for dichotomous outcomes. The 5% level often used for continuous outcomes was judged to be too low for measuring a biologically significant increase in motor activity. Conclusions were consistent across the analyses.

Analysis of the USN Study (Bekkedal et al., 2000):

Female Sprague-Dawley rats were dosed with ammonium perchlorate for two weeks at 0, 0.1, 1.0, 3.0 or 10.0 mg/kg-day prior to mating with the breeder males and through PND10. PND1 was counted as the day when the first pup was observed in the cage. All pups within a litter were weighed on PND5, when the litters were culled to eight pups of 4 males and 4 females, or as close as possible to that combination. Pups and dams from any litters with less than 8 pups were eliminated. On PND14, one male and one female were randomly selected from each litter to be used in the motor activity testing. These same animals were tested on PND14, PND18 and PND22.

Nine different measures of motor activity were automatically recorded using Opto-Varimex activity meters at ten minute intervals. The measures included: frequency and time of ambulatory movements, frequency and time of sterotypic movements, frequency of movements in the horizontal plane, distance traveled in the horizontal plane, frequency of rears, total number of horizontal movements made while in the rearing position (vertical plane movements) and time spent resting. Since the correlation between these different measures was extremely high, we focused our analysis on the number of ambulatory movements.

We found that the effect of ammonium perchlorate on the number of ambulatory movements increases significantly with habituation time (posterior probability = 0.98). In the first habituation interval there was modest evidence of an increase in motor activity with dose (posterior probability = 0.79), while in the final interval there was clear evidence of an increase in motor activity with dose (posterior probability > 0.99). The dose estimated to increase the mean number of ambulatory movements at the final habituation time by 10% is 1.62 with a 95% credible interval of (0.90, 7.87). There was no evidence of an interaction between age and dose, nor of any effect of gender.

Analysis of the Argus (1998) Study:

Motor activity in the Argus (1998) study was evaluated on pups at PND14, 18, 22 and 59. Because Argus Laboratories designate the day of birth as PND1, these days correspond with the convention used in the USN study . In contrast to the USN study, dosage began

at the first day of gestation and continued through parturition and up to lactation day 10 (PND10). Dams were dosed at 0, 0.1, 1.0, 3.0 and 10.0 mg/kg-day. Movements of each pup were monitored by a passive infrared sensor. Each test session was 90 minutes in duration. The number and time spent in movement was tabulated at each five-minute interval. In order to be comparable with the USN analysis, every two of the five-minute intervals were combined into a ten-minute interval. However, the USN study did not have data for PND 59, so the results are not entirely comparable.

We used the same approach to analyze data from the Argus (1998) study, as was used in analyzing the Navy data, and the results were very similar. Again, there was evidence of an increase in the effect of ammonium perchlorate on motor activity at the later habituation times (posterior probability = 0.93). In the first habituation interval there was no evidence of an increase in motor activity with dose (posterior probability = 0.58), while in the final interval there was moderate evidence (posterior probability = 0.94). The dose estimated to increase the average of ambulatory movements in the final habituation time by 10% is 4.60 with a credible interval of (2.18, infinity). This interval was wider than the interval observed in the USN study, possibly due to greater variability in the Argus data (as noted earlier by EPA).

Analysis of Combined Data:

Within the Bayesian framework, it is natural to combine data from different studies. Therefore, we chose to perform a combined analysis using the approach described in Appendix A. This approach allows the different studies to have distinct baseline parameters, including aging effects.

The posterior densities for the expected increase in the logarithm of the number of ambulatory movements at the final habituation time per unit (mg/kg/day) increase in dose of ammonium perchlorate are plotted in Figure 1 for the Argus and USN studies. Both of the posterior densities are centered on positive slopes and assign low probability to negative slopes, suggesting a clear increase in motor activity with dose.

Figure 2 shows the posterior density from the combined analysis of the Argus and USN study. In this combined analysis, the posterior probability of an increase in motor activity with dose was 0.99. For rats that average 34.09 ambulatory movements at the final habituation time in the absence of exposure (the average value in the Argus study), the esimated dose needed to increase this average by 10% is 3.33 [95% credible interval = (1.91,12.78)].

Summary:

There was evidence of an increasing dose-response trend in motor activity in both the Argus and USN studies, though the effect in the Argus study was less pronounced.

Our Bayesian analysis can be applied to risk assessment in an analogous fashion to the benchmark dose analysis of Hasselblad and Jarabek (1996). The lower limit on the estimated dose corresponding to a 10% increase in motor activity relative to control can be used as a surrogate for the NOAEL level for the point of departure for reference dose derivation. For the Argus study, the lower limit of the 95% credible interval for the dose was 2.18, while for the USN study the corresponding estimate was 0.90. In the combined analysis, the lower limit was 1.91. Therefore, a reference dose of 1-2 represents a conservative estimate.

Appendix A: Statistical Model for Analyzing Motor Activity Data

Let *i* index the animal, let *j* index the testing age, and let *k* index the habituation interval. The following model was used as a basis for our analyses of motor activity:

$$E(y_{ijk}) = B_1 + B_2 dose_i + B_3 sex_i + Lb_i + G_j + Adose_I \times \log(k-1),$$

where y_{ijk} is the number of ambulatory movements,
B_1, B_2, B_3 are regression coefficients,
$dose_i$ is the dose of ammonium perchlorate,
sex_i =1 if the animal is male and 0 otherwise,
b_i is a standard normal animal-specific variable,
L is a factor loading parameter,
G_j is a age category parameter (G_0=0),
$Adose_I \times \log(k-1)$ models the effect of habituation time.

We also considered models that allowed for dose-age and dose-gender interactions. Finding no evidence of such interactions, we focused on an appropriately simplified model. The term adjusting for an interaction between dose and habituation time provided a good fit to the observed data. In the combined analysis, we allowed distinct baseline parameters (B_1, B_3, L, G_j) for the two studies.

Following a Bayesian approach, we assigned prior distributions to each of the unknown parameters in the above model. We then updated these priors, which were chosen to be essentially non-informative, by incorporating the likelihood of the data. Posterior summaries of the parameters were obtained by using Gibbs sampling within the BUGS software package (Gilks et al., 1994). Estimates of the dose corresponding to a 10% increase in risk were obtained by straightforward inverse estimation. Due to the well known Fieller problem, this dose is undefined when the slope is less than or equal to 0. Therefore, when the lower bound on the credible interval for the slope is negative, we follow the approach of setting the upper bound on the dose equal to infinity.

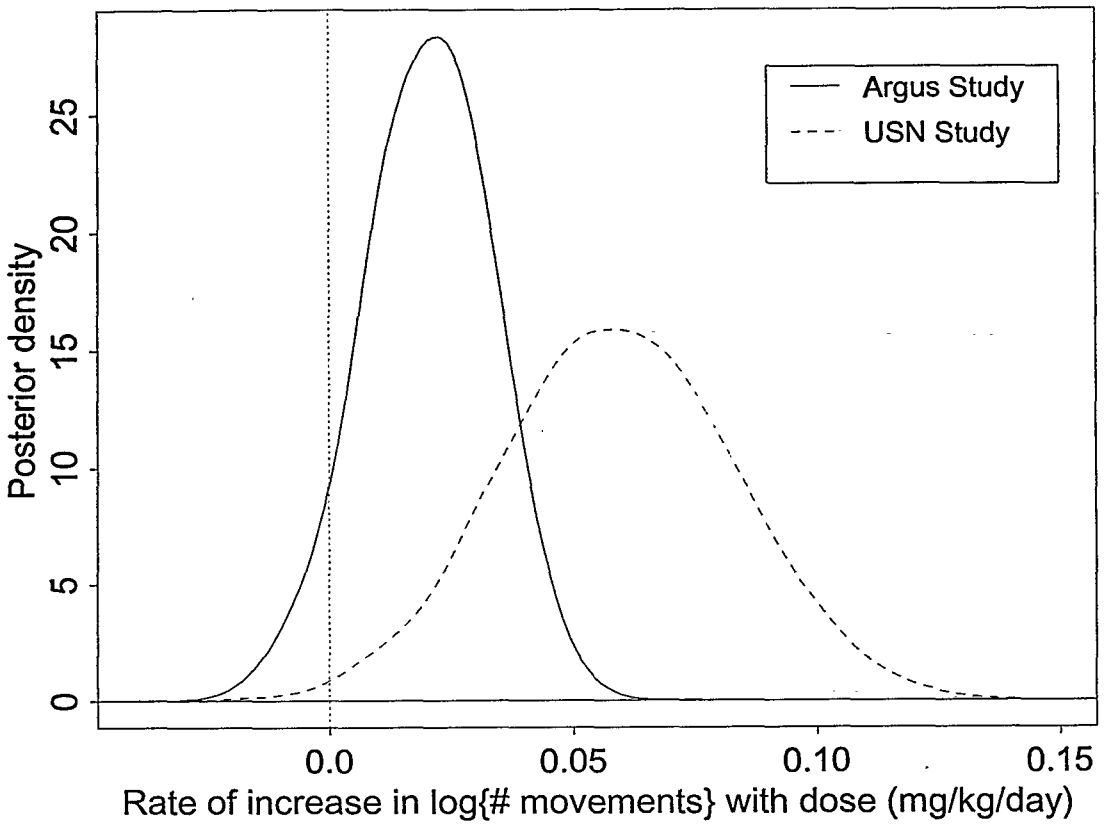

Fig 1 Estimated posterior densities for the expected increase in the logarithm of the number of ambulatory movements at the final habituation time per unit (mg/kg/day) increase in dose of ammonium perchlorate (SEPARATE ANALYSIS FOR USN AND ARGUS).

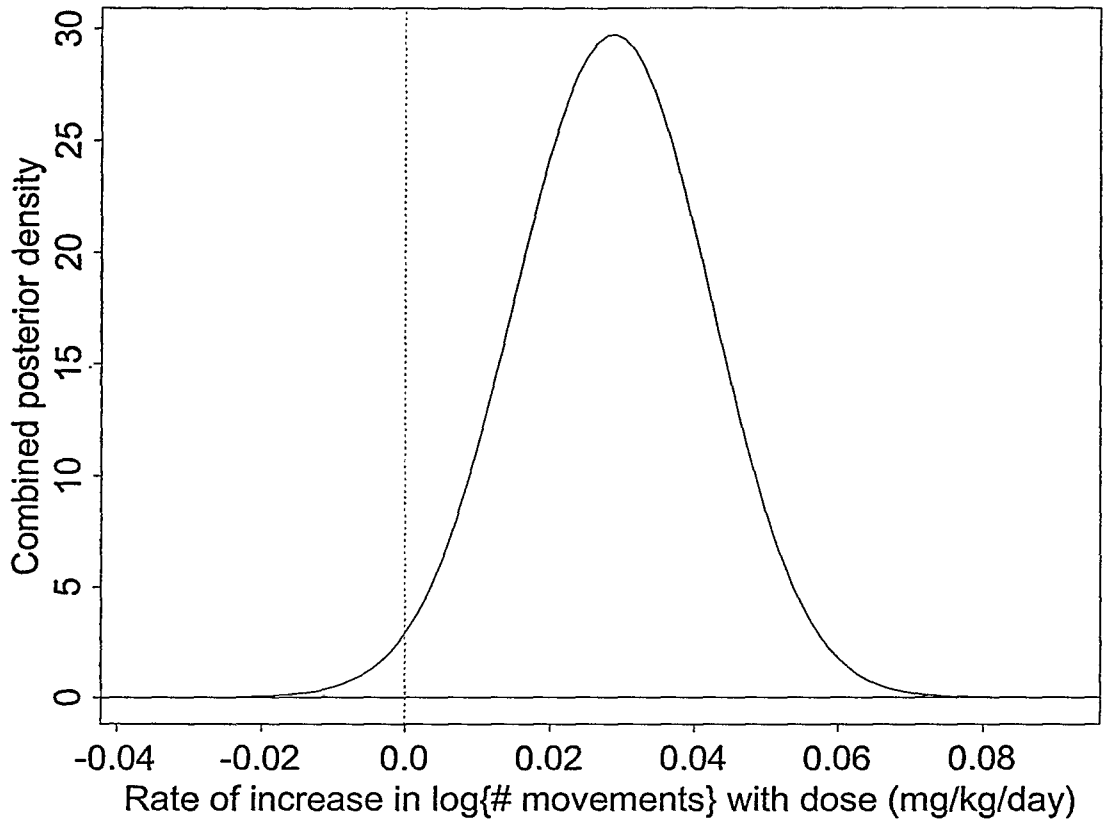

Fig 2 Estimated posterior densities for the expected increase in the logarithm of the number of ambulatory movements at the final habituation time per unit (mg/kg/day) increase in dose of ammonium perchlorate (COMBINED ANALYSIS).

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